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(54) Title: FUNGICIDALLY ACTIVE PREPARATIONS

(57) Abstract

Microbial enzyme preparations having fungicidal activity comprising an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure, compositions comprising said preparations, and synergistic preparations further comprising a conventional fungicide.

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Title: FUNGICIDALLY ACTIVE PREPARATIONS

FIELD OF THE INVENTION

The present invention relates to the use of specific enzymes for controlling and combating fungi, fungicidal compositions comprising said enzymes alone or in combination with other fungicidally active agents, and methods for combating fungi by applying said compositions - the enzyme complex alone or in combination with other fungicidally active agents.

PRIOR ART

The use of lytic enzymes in plant protection has previously been described.

Enzyme preparations produced by fermentation of bacteria (Bacillus) and used as food preservatives and as plant protecting fungicides have been disclosed in Japanese Patent Publication No. 77 139 423. The microbe-lytic activity is here described to be caused primarily by β-1,3-glucanase, protease, and lysozyme-like enzymes.

Herbicidal, insecticidal and fungicidal use of enzymes to degrade protective or supporting components of the organism to be controlled has likewise been disclosed in European Patent Publication No. 184288.

A further patent publication describing the use of enzymes within the field of biocides is European Patent Publication No. 197622, which describes the use of esterases for enhancing the effect of a biocide by applying the esterase to the plant stem, leaves or an insect prior to or together with the application of the biocide. The esterases described all come from bacteria of the genus Pseudomonas, Colletotrichum, or Enterobacterium, or Botrytis fungi.

Also European Patent Publication No. 272002 describes enhancing the effect of agricultural chemicals by treating a plant with a plant depolymerase enzyme that will degrade plant surface polymers either prior to or concurrently with applying the agricultural chemical. The enzymes descri-

bed are more or less the same as in EP 197622, such as lipases, pectinases, hemicellulases, cellulases or proteinases.

Laboratory experiments with in vitro systems have suggested the possibility of lowering MIC-values (Minimal Inhibitory Concentration) of chitin biosyntheses inhibiting fungicides by the combined use of non-ionic detergents and enzyme complex of Bacillus circulans WL12 against Pyricularia oryzea (Watanabe et al. Agric. Biol. Chem., 52, 895-901 (1988)). In order to obtain a similar effect on the mycelium of Glomerella cingulata and Alternaria kikuchiana (as scored by microscopic obsevation of in vitro growth of the mycelium) Watanabe et al. (supra) found it necessary to add enzyme complex Novozym 234, produced by Trichoderma harzianum, or crude enzyme preparation from Streptomyces sp. They interpreted the presence of mannase and/or protease to be the key factor.

The microbial antagonistic effect of species belonging to the fungal genus Trichoderma is a well established fact known and described in numerous scientific papers and text books for decades. The role of cell wall lysing enzymes in this antagonistic activity of Trichoderma species has recently been described in a comprehensive investigation by Jacobs & Kamoen [Med. Facult. Landbw. Ricks Univ. Gent, 51: 751-758, 1986)]. But already in 1982 Huttermann & Cwienlong [Eur. J. Forest Path., 12: 238-245] illustrated the role of enzymes in the antagonism of Trichoderma species by demonstrating the lysis of cell wall of Fomes annosus (a Basidiomycete) due to lytic enzymes produced by Trichoderma harzianum.

Further understanding of the activity of the complex of enzymes produced by T.harzianum has been achieved by the work of Ridout, Coleysmith & Lynch; Enzyme and Microbial Technology 10: 180-187 (1988) in which they succeeded in fractionating the extracellularly produced enzymes. Special focus was put on the role of chitinase in biocontrol of Sclerotium rolfsii by Ordentlich, Elad & Chet; Phytopathology 78: 84-88 (1988).

The potential of Trichoderma harzianum as biocontrol agent has been examined, investigated and evaluated for many

years. T.harzianum has been demonstrated to have effect against both Phycomycetes [Pythium: Hadar, Harman & Taylor; Phytopathology, 74: 106-110 (1984)], Ascomycetes [Botrytis: Tronsmo; Vakstskyddsnotiser, 45, 66-72 (1983)], Fusarium [Silvan & Chet; British Crop Protection Conference. Pests and Diseases, 2: 865-872 (1986), US Patent No. 4,713,342 and European Patent Publication No. 133878] and Basidiomycetes, Rhizoctonia solani [Lewis & Papavizas; Phytopathology 77: 699-703 (1978)]. Special attention has been focused on the level of competition of T.harzianum in the rhizosphere of the plants [Ahmad & Baker; Phytopathology 77: 182-189 (1987)] and the feasibility of applying T.harzianum directly to the seed surface to protect against damping-off [Chao, Nelson, Harman & Hoch; Phytopathology 76: 60-65 (1986)].

Use of Trichoderma species, especially T.harzianum, T.polysporum, and T.viride for controlling harmful fungi is further described in German Patent Publication No. 3600394. Said patent publication is especially related to the treatment of wood or timber.

However, T.harzianum has only in rare cases been demonstrated to be effective enough when applied as the only control measure. Therefore attempts have been made to elucidate the possibilities of integrated control strategies using both T.harzianum and chemical fungicides [Kraft & Papavizas; Plant Disease 67: 1234-1237 (1983), Vargas; Agronomia Costrarricense 8: 91-97 (1984), Strashnow, Elad, Sivan & Chet; Plant Pathology 34: 164-151 (1985), and US Patent No. 4,713,342]. Another approach to the improvement of the efficacy of biocontrol through the use of T.harzianum has been to obtain improved strains of the fungus through protoplast fusion [Stasz & Harman; Phytopathology 77: 1771 (1987)].

The use of fermented products produced by submerse culturing of T.harzianum outside the field of plant protection is represented by US Patent No. 4,439,455 relating to the clarification of wine, and US Patent No. 4,353,891 relating to plaque treatment of teeth.

The use of e.g. vitamins, co-enzymes or fatty acids as additives to agrochemicals are claimed in GB 2030452 to

enhance the efficacy of such chemicals. However, similar use of enzymes giving a synergistic effect in vivo has not previously been disclosed.

DESCRIPTION OF THE INVENTION

From the publications summarised above it appears that lytic enzymes have been described to be of use in plant protection and that Trichoderma species are well-known to have an effect as inhibitors of fungal growth when applied directly as biocontrol agents. It is also described that part of the antagonistic effect of Trichoderma species is due to an enzymatic effect.

However, it has never before been demonstrated that this enzyme complex may be recoverede from the culture broth, purified and used alone or in combination with fungicidally active agents, without the application of the microorganism itself.

The present invention reports for the first time the use of lytic enzymes as fungicides and as additives to other fungicides obtaining improved disease control in plants. It has here been demonstrated that a synergistic effect of the fungicidally active lytic enzyme complex and another fungicidally active compound or composition is often obtained. This synergistic effect allows application of the other fungicide in considerably lower dosages than the ones usually applied while still retaining the same or improved control effect of the fungal pathogen.

When the enzymatic preparation is used in combination with a fungicide, as well as reducing the amount of fungicide required to control a particular pathogen, the combination can sometimes broaden the spectrum of activity of the fungicide and/or increase the level of control by the fungicide of a particular species, to a more desirable level. For example improved control of Erysiphe graminis by the fungicide prochloraz was obtained level when applied in mixture with the enzyme preparation; and similarly improved control of potato late blight by the fungicide propamocarb was shown when used in the presence of the enzyme preparation.

The invention is primarily applicable to fungal plant pathogens which develop a significant part of their thallus outside the plant. This covers primarily species of the groups powdery mildew, downy mildew, grey mold and collar rot. Implicit in this statement lies the fact that the invention is valid for species of both Phycomycetes, Actinomycetes and Basidiomycetes.

The invention thus also provides a method of combating fungi at a locus infested or liable to be infested therewith, which comprises applying to the locus a fungicidally active preparation of the invention, and in a preferred embodiment of the invention said locus is subjected sequentially or in a mixture to the enzyme preparation of this invention as well as said other fungicide.

The invention also provides an agricultural composition comprising the enzyme preparation of the invention and a fungicide in admixture with an agriculturally acceptable diluent or carrier.

Examples of other fungicides which can be combined with the enzyme preparation of the invention include especially ergosterol biosynthesis inhibitors ("EBIs"). These are generally imidazole or triazole derivatives and examples include those known by the common names prochloraz (which is particularly preferred), triadimefon, propiconazole, diclobutrazol, triadiminol, flusilazole, flutriafol, myclobutanil, penconazole, quinconazole, imazalil and diniconazole. Examples of non azole EBis include nuarimol, fenarimol, fenpropimorph, tridemorph and fenpropidine. Other fungicides which can be combined with the enzyme preparation of the invention include anilides, e.g. carboxin, matalaxyl, furalaxyl, ofurace, benalaxyl, mepronil, flutolanil, pencycuron and oxadixyl; benzimidazoles, e.g. benomyl and carbendazim; carbamates, e.g. maneb, mancozeb and propamocarb; dicarboximides, e.g. iprodione, vinclozolin and procymidone; phosphorus derivatives, e.g. pyrazophos, tolclofos-methyl and fosetyl aluminum; and miscellaneous compounds, including iminoctadine, guazatine, dicloran, chlorothalonil, pyrifexox, ethirimol, cymoxanil and anilazine.

The diluent or carrier in the compositions of the invention can be a solid or a liquid optionally in association with a surface-active agent, for example a dispersing agent, emulsifying agent or wetting agent. Suitable surface-active agents include anionic compounds such as a carboxylate, for example a metal carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono- and di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulphates such as sodium dodecyl sulphate, sodium octadecyl sulphate or sodium cetyl sulphate; ethoxylated fatty alcohol sulphates; ethoxylated alkylphenol sulphates; lignin sulphonates; petroleum sulphonates; alkyl-aryl sulphonates such as alkyl-benzene sulphonates or lower alkyl-naphthalene sulphonates, e.g. butyl-naphthalene sulphonate; salts of sulphonated naphthalene-formaldehyde condensates; salts of sulphonated phenol-formaldehyde condensates; or more complex sulphonates such as the amide sulphonates, e.g. the sulphonated condensation product of oleic acid and N-methyl taurine or the dialkyl sulphosuccinates, e.g. the sodium sulphonate of dioctyl succinate. Nonionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, e.g. sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, e.g. polyoxyethylene sorbitan fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetraethyl-5-decyn-4,7-diol, or ethoxylated acetylenic glycols.

Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamine as an acetate, naphthenate or oleate; an oxygen-containing amine such as an amine oxide or polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

The compositions of the invention can take any form known in the art for the formulation of agrochemicals, for

example, a solution, a dispersion, an aqueous emulsion, a dusting powder, a seed dressing, a dispersible powder, an emulsifiable concentrate or granules. Moreover it can be in a suitable form for direct application or as a concentrate or primary composition which requires dilution with a suitable quantity of water or other diluent before application.

An emulsifiable concentrate comprises the active ingredient dissolved in a water-immiscible solvent which is formed into an emulsion with water in the presence of an emulsifying agent.

A dusting powder comprises the active ingredient intimately mixed and ground with a solid pulverulent diluent, for example, kaolin.

A granular solid comprises the active ingredient associated with similar diluents to those which may be employed in dusting powders, but the mixture is granulated by known methods. Alternatively it comprises the active ingredient absorbed or adsorbed on a pre-granular diluent for example, Fuller's earth, attapulgite or limestone grit.

Wettable powders, granules or grains usually comprise the active ingredient in admixture with a suitable surfactant and an inert powder diluent such as china clay.

Another suitable concentrate is a flowable suspension concentrate which is formed by grinding the active ingredient with water or other liquid, a wetting agent and a suspending agent.

The concentration of the fungicidally active enzyme preparation in the compositions of the present invention when used alone or in combination with a conventional fungicide, as applied to plants is preferably within the range from about 0.01 to about 3.0 per cent by weight, especially 0.01 to 1.0 per cent by weight. In a primary composition the amount of active enzyme preparation can vary widely and can be, for example, in the range from about 5 to about 95 per cent by weight of the composition.

The concentration of the other fungicidally active ingredient in the mixed composition of the present invention, as applied to plants is preferably within the range of 0.001

to 10 per cent by weight, especially 0.01 to 5 per cent by weight. In a primary composition the amount of active ingredient can vary widely and can be, for example, from 5 to 80 per cent by weight of the composition.

The active enzyme preparation or the compositions of the invention can be applied directly to the plant by, for example, spraying or dusting either at the time when the fungus has begun to appear on the plant or before the appearance of fungus as a protective measure. In both such cases the preferred mode of application is by foliar spraying. It is generally important to obtain good control of fungi in the early stages of plant growth as this is the time when the plant can be most severely damaged. The spray or dust can conveniently contain a pre- or post-emergence herbicide if this is thought necessary.

Sometimes, it is practicable to treat the roots of a plant before or during planting, for example, by dipping the roots in a suitable liquid or solid composition. When the active enzyme preparation of the invention is applied directly to the plant a suitable rate of application is from 0.01 to 10 kg per hectare, preferably from 0.05 to 5 kg per hectare.

In the method of the invention the active enzyme preparation of the invention alone or in combination with a conventional fungicide can also be applied to seeds or habitat. Thus the preparation can be applied directly to the soil before, at or after drilling so that the presence of active ingredient in the soil can control the growth of fungi which may attack seeds.

When the soil is treated directly the active enzyme preparation alone or in admixture with the conventional fungicide can be applied in any manner which allows it to be intimately mixed with the soil such as by spraying, by broadcasting a solid form of granules, or by applying the active ingredient at the same time as drilling by inserting it in the same drill as the seeds. A suitable application rate is within the range of from 0.05 to 20 kg per hectare, more preferably from 0.1 to 10 kg per hectare.

The invention is illustrated in the following exam-

ples:

Example 1

The enzyme complex from T.harzianum, produced as indicated in Example 1 in US Patent No. 4,353,891, which is hereby incorporated in its entirety for reference, was assessed for activity against Erysiphe graminis tritici: wheat powdery mildew on detached wheat leaves. For convenience the preparation obtained is designated P1 in the following parts of this specification.

21 day old wheat plants, cv. Armada, were inoculated with conidia of E.graminis and incubated for 3 days in the glasshouse. 3 cm lengths of the first leaf were detached from the plants and placed on benzimidazole agar (150 ppm benzimidazole + 0.5% w/v agar) in clear plastic boxes (3 leaf sections per box).

The enzyme preparation, P1 (which is a mixture of chitinase, gluconase, mutanase, protease, esterase etc.) was dissolved in distilled water containing wetters to give solutions containing various concentrations of the enzyme. The solutions were painted onto upper surfaces of the detached leaves.

Immediately following treatment with the enzyme solutions the same leaves were painted with 5 or 1 ppm solutions of prochloraz. Appropriate control treatments were also included, each treatment being replicated 5 times.

The test boxes were incubated for a further 3 days (15-20°C) following which the level of infection on each leaf was determined on a 0-5 scale (where 0 = no infection and 5 = 100% infection). The results are summarised in Table 1.

Table 1:

Treatment	% control
P1 10,000 ppm	88
P1 10,000 ppm plus 5 ppm prochloraz	98
P1 10,000 ppm plus 1 ppm prochloraz	98
P1 1,000 ppm	79
P1 1,000 ppm plus 5 ppm prochloraz	95
P1 1,000 ppm plus 1 ppm prochloraz	96
prochloraz 5 ppm	89
prochloraz 1 ppm	71
wetter controls	55

This example clearly demonstrates the fungicidal activity of the enzyme preparation of the invention and also the enhancement of the activity of prochloraz obtained by the addition of the P1 preparation.

Example 2

Technical prochloraz was dissolved in acetone plus Tween 20 to give solutions of 1,000, 500, 250 and 125 ppm prochloraz plus 125 ppm Tween. Pluronic L 61 was also added to each to give a final concentration of 0.1% v/v.

100 ml aliquots of each solution were removed and 0.1 g of P1 was added to each (equivalent to 1,000 ppm P1). Separate, 1,000 ppm solutions of P1 were also prepared, either on its own or with the wetters.

These solutions were sprayed onto 21 day old wheat plants, cv Armada, using a calibrated track sprayer (2 x 8004 nozzles at 1 m sec^{-1}, 2 bar pressure). The plants had been inoculated with E.graminis 3 days before treatment. The application rates used were equivalent to 400, 200, 100 and 50 g ap ha^{-1} (active preparation pr hectare) prochloraz. Each treatment was replicated 5 times. The treated plants were then returned to the glasshouse. Disease levels were determined on leaf 1 at 3 and 7 days after treatment. the results are summarised in Table 2.

Table 2:

Treatment	Rate (g ai/ha)	Conc. P1 (ppm)	% control E.graminis 3 DAT	7 DAT*
prochloraz	400	-	82	82
	200	-	89	74
	100	-	86	49
	50	-	59	33
prochloraz +	400	1,000	89	95
P1	200	1,000	95	77
	100	1,000	91	72
	50	1,000	82	46
P1	-	1,000	17	10
P1 + wetters	-	1,000	38	18
wetters only	-	-	13	23
water only	-	-	1	8

*) DAT = days after treatment

From Table 2 it is seen that the P1 enzyme preparation has a synergistic effect on the control in combination with Prochloraz.

Example 3

Aqueous dispersions of various fungicides at various concentrations were sprayed over pots each containing about seven wheat seedlings, cv Armada, which had been inoculated with E.graminis 2 days before treatment. Eight pots were used for each dose of each fungicide. Half the pots were then sprayed with 10,000 ppm solutions of P1 containing wetters. Eight pots were also each sprayed with 10,000 ppm solutions of P1 containing wetters, and a further eight pots with 10,000 ppm solutions of P1 without wetters. Plants were then kept under controlled environment conditions suitable for maintaining plant growth and development of the disease. After seven days, the degree of infection of the leaf surface was visually estimated. The results are summarised in Table 3.

Table 3:

Treatment	Rate (ppm)	% control E.graminis with P1	without P1
prochloraz	1	97	79
	0.5	94	69
cyproconazole	1	100	100
	0.5	94	82
flusilazole	1	94	88
	0.5	94	85
triadimenol	1	97	79
	0.5	94	88
fenpropimorph	1	94	57
	0.5	97	39
flutriafol	1	100	85
	0.5	85	76
P1	10,000	57	
P1+ wetters	10,000	57	

Similarly to Example 3, also this Example reveals a marked synergistic effect of the enzyme preparation of the invention.

Example 4

Potato plants (20 days old, planted individually in 8 cm plastic pots) were sprayed with metalaxyl at a rate of 10^{-6}ppm and P1 at a rate of 0.1% alone and in combination. The plants were then incubated for 24 hrs at 18°C after which they were inoculated with aqueous suspension of Phytophthora infestans sporangia. Following inoculation, the potato plants were incubated in darkness in humid chambers at 16-18°C for 24 hrs. For the following 48 hrs the plants were retained in the humid chambers but provided with light 16 hrs per day. At days 4-10 the plants were kept in the growth chambers at 16-18°C with light and without plastic cover. The percentage control was assessed 8 days after the inoculation, and the results are shown in Table 4.

Table 4.

Treatment	% control
metalaxyl	0%
P1	25%
metalaxyl + P1	50%

Example 5

Propamocarb and P1

The enzyme preparation P1 was weighed and dissolved in distilled water/wetters to give the desired concentration.

The resulting solution was sprayed, alone or in combination with propamocarb, onto eight replicate potato plants, which were inoculated 24 hrs later with a suspension of sporangia and zoospores of P.infestans. Plants were incubated for 5 days in humid conditions at approx. 18°C and then assessed for disease symptoms.

Results

P1 alone at 1,000 ppm (0.1%) gave moderate disease control (Table 5). Propamocarb at 1,500 ppm was more active. The two components combined gave a level of disease control greater than that of propamocarb alone, and with both components at half rate disease control was approx. equal to that of propamocarb at the high rate.

Table 5

Treatment	ppm	% disease control
P1	1,000	55
Propamocarb	1,500	79
Propamocarb + P1	1,500) 1,000)	92
Propamocarb + P1	750) 500)	83

This Example shows clearly the synergistic effect

between the enzyme preparation of the invention and a conventional fungicide.

C L A I M S

1. The use of an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure as a fungicide.

2. The use of an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure as a fungicide in combination with another fungicide.

3. The use of an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure as an additive to another fungicidally active preparation.

4. An agricultural composition comprising an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure in admixture with an agriculturally acceptable diluent or carrier.

5. A composition according to claim 4, further comprising at least one further fungicidally active agent.

6. A composition according to any of the claims 4 or 5, wherein said enzyme preparation comprises a single enzyme.

7. A composition according to any of the claims 4 or 5, wherein said enzyme preparation comprises a mixture of enzymes.

8. A composition according to any of the claims 4 to 7, wherein said enzyme preparation comprises enzymes produced by microorganisms.

9. A composition according to any of the claims 4 to 8, wherein said enzyme preparation comprises one or more enzymes selected from the group comprising chitinases, gluconases, mutanases, proteases, and esterases.

10. A composition according to any of the claims 4 to 9, wherein said enzyme preparation is produced by culturing Trichoderma sp.

11. A composition according to claim 10, wherein said enzyme preparation is produced by culturing Trichoderma harzianum.

12. A composition according to any of the claims 5 to 11, wherein said other fungicidally active species is chosen from the group comprising ergosterol biosynthesis inhibitors (azoles or non-azoles), anilides, benzimidazoles, carbamates, dicarboximides, and phosphorous derivatives.

13. A composition according to claim 12, wherein said other fungicidally active species is a carbamate or an ergosterol biosynthesis inhibitor anilide, preferably an ergosterol biosynthesis inhibitor anilide.

14. A composition according to claim 13, wherein said other fungicidally active species is prochloraz.

15. A method of combating fungi at a locus infested or liable to be infested therewith, which comprises applying to said locus an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium, fungal spores or other vital parts of a fungal structure, or a composition according to any of the claims 4 to 14.

16. A method of combating fungi at a locus infested or liable to be infested therewith, which comprises applying to said locus an enzyme preparation capable of degrading the cell wall or parts of the cell wall of fungal mycelium,

fungal spores or other vital parts of a fungal structure, or a composition according to claim 4 in combination with at least one other fungicidally active preparation.

17. A method according to claim 16, wherein said at least two preparations are applied simultaneously.

18. A method according to claim 16, wherein said at least two preparations are applied sequentially.

19. A method according to any of the claims 16 to 18, to control plant pathogenic and storage fungi.

20. A method according to any of the claims 16 to 19, to control fungi which attack aerial plant parts.

21. A method according to any of the claims 26 to 20, to control grey mold, powdery mildew and downy mildew.

22. A method according to any of the claims 16 to 21, to control attacks from fungal species belonging to the genera Erysiphe and Phytophthora.

23. A method according to claim 22, to control Erysiphe graminis and potato late blight (Phytophthora infestans).

24. A method according to claim 23, to control attacks on cereal crops, especially wheat.